ORIGIN OF DNA AND PROTEIN IN λ DNA INFECTED DISRUPTED CELL PREPARATIONS

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SUMMARY: By employing the density labels $^{15}NH_4Cl$ and D_2O to prepare bacteria and D_2O to prepare λ bacteriophage, the de novo synthesis of viral DNA and protein in frozen-thawed cell preparations has been demonstrated.

We have previously described frozen-thawed preparations from virus-resistant E. coli cells which can be infected by isolated λ DNA.[1-3]. However, the amount of virus production was insufficient to demonstrate net DNA synthesis. Consequently the possibility could not be excluded that DNA was not being synthesized, but rather that portions of the infecting DNA were simply encapsulated in newly synthesized virus protein. By employing density labels $^{15}NH_4Cl$ and D_2O to prepare bacteria and D_2O to prepare λ bacteriophage, de novo synthesis of viral DNA and protein in the frozen-thawed bacterial preparations has been demonstrated.

MATERIALS AND METHODS

Preparation of density labeled phage and bacterial cells—Unlabeled stocks of phage λ temperate and λ virulent were prepared and purified as previously described.[4] Density labeled phage was prepared by substituting D_2O (99.86%) for H_2O in the Fraser medium.* 1900 ml batches of medium in 4 liter Erlenmeyer flasks were inoculated with 100 ml cultures of E. coli W-1485 grown overnight at 37°. The 2 liter cultures were incubated with aeration at 37° to give a final concentration of 1×10^9 cells/ml. The dry air used for aeration was first passed through a bacterial filter and a trap containing D_2O to prevent contamination and excessive evaporation. 4×10^{12} λ virulent phages (multiplicity of 2) were added and incubation was continued until lysis was complete (4 to 6 hrs). The crude lysates contained a minimum of 3×10^{10} plaque forming units/ml. The crude labeled lysates were purified in the same way as unlabeled lysates,[4] except that only the heavy phage band was removed from the CsCl density gradients. Traces of light unlabeled phage appearing above the

* $^{15}NH_4Cl$ cannot be used with Fraser medium for labeling purposes due to the presence of large amounts of other nitrogen-containing compounds.

major phage band were occasionally observed as a result of the reisolation of the small fraction of unadsorbed phage inoculum.

Frozen-thawed bacterial cells of E. coli 3350/λ,1,7 were prepared by a slight modification of the method previously described.[1] The medium described by Fangman and Feiss,[5] prepared with D_2O and $^{15}NH_4Cl$ (99.86% D and 99% ^{15}N, International Chemical and Nuclear Corp.), was used. Inoculated 1 liter batches of this medium were incubated overnight with shaking at 37° in 2.5 liter Fernbach flasks. The cell cultures were centrifuged at 6,000 x 6 for 10 min in a Sorvall angle centrifuge. The supernatant solution was discarded and each tube drained by inversion. The cell paste was then applied with a small spatula to stainless steel blocks kept at a temperature of -40° to -35°. The frozen cells were then ground in a small mortar and kept at a temperature below -40° with powdered dry ice. The ground cells were stored at -80°. Unlabeled cells were prepared identically except that the growth medium was prepared from non-isotopic components.

DNA and phage density comparison—Aliquots (15 μg) of λ DNA preparations were added to tubes containing Tris buffer (0.067 M Tris, 0.5% NaCl and 0.025% $MgSO_4$ at pH7) and 5.3 g CsCl per tube to give a final volume of 5.5 ml. The tubes were centrifuged in a Spinco SW 50 L rotor for 24 hrs at 44,000 rpm. The contents of each tube were then passed through an ISCO density gradient fractionator 180 and the absorbancy profile at a wavelength of 254 nm was recorded.

For comparison of phage densities, suitable aliquots were added to centrifuge tubes containing Tris buffer and 620 to 680 mg CsCl/ml depending on the densities required to position the phage concentrations and the density beads. Beckman-Spinco density beads, 1.4, 1.5 and 1.6 nominal density, were included in each tube. The final volume in each tube was 5.5 ml. All samples were then centrifuged for 20 hrs at 39,000 rpm in the Spinco SW 50 L rotor. The contents of the centrifuged tubes were fractionated by collecting fractions of either 2 or 5 drops. Each fraction was then assayed for phage by standard agar plating techniques.

Preparation of infectious DNA—Infectious DNA was prepared by a modification of the Gierer and Schramm method.[6] 0.5 ml of stock solutions of phage in Tris buffer containing 2×10^{13} plaque forming/ml were added to 2.5 ml of Tris buffer and 5.0 ml of water-saturated phenol, pH 7. All solutions were kept at 4° throughout the procedure. The preparation was then gently agitated for 5 min by a rolling movement. After centrifugation of the mixture at 12,000 x g for 10 min in a Sorvall centrifuge, the DNA layer was removed by means of a wide mouth Pasteur pipet (mouth orifice 2 mm in diameter) and placed in dialysis bags. The preparations were dialyzed four times in 1 liter volumes

of Tris buffer at 4°. Absorbancy of 0.02 per μg of DNA at 260 nm was used to estimate the quantity of DNA in each preparation. All DNA preparations were plated directly on indicator bacteria prior to use. The absence of plaque formation in every case indicated that no contaminating viable phage was present in the DNA preparations.

Infection of frozen-thawed cell pastes—Fifteen mg of Difco casamino acids were added to round, flat bottomed vials, 2 cm diameter, 5 cm height, containing 100 mg of ground frozen cells, labeled or unlabeled as required, plus .05 ml (25 μg DNA) of appropriate DNA preparations. The samples, including the appropriate control (vial containing cell paste and amino acids but no DNA) were incubated for 70 min with gentle shaking in a 37° water bath. A 1 ml aliquot of Tris buffer was added and each tube was shaken for 45 sec at 20° with a Vortex test tube mixer at maximum speed (Scientific Industries, Inc., Model K 500-4). Each mixture was then added to 39 ml of density labeled medium and incubated for 3 hrs at 37°. The incubated material was centrifuged at 6,000 x g for 10 min in an angle centrifuge and the supernatant solution, after removal, was subjected to 30 min of centrifugation at 39,000 rpm in a Spinco No. 50 rotor. The supernatant solutions were assayed for plaque forming units by standard plating techniques and appropriate quantities applied to CsCl gradients as required. When experiments were conducted with density labeled infectious DNA, all materials used consisted of non-isotopic components.

As controls, aliquots of all preparations of resistant frozen-thawed cells were mixed with intact virus (10^{12} plaque forming units per plate). The absence of plaque formation in every case indicated that the cells were completely resistant to the virus. In each experiment the absence of viral contamination was established by subjecting a tube containing frozen-thawed cells, but without DNA, to all the experimental operations employed in the case of DNA infected frozen-thawed cell pastes, with subsequent plating on indicator bacteria. The absence of plaques under these conditions again indicated that contamination by extraneous virus particles was absent.

Density measurements of phage added to the density labeled incubation medium and incubated for 3 hrs were also made. Such phage was indistinguishable from unlabeled phage indicating that phage density was not increased by exchange of D_2O or $^{15}NH_4Cl$.

RESULTS AND DISCUSSION

As can be seen in Fig. 1A, when λ temperate DNA infected frozen-thawed cells and incubation medium are density labeled, the newly formed phage is also heavily labeled. If initially the incubation medium only is labeled, the synthesized phage is still heavily labeled (Fig. 1B). As shown in Table I, the relative density difference in these two cases indicates that 80% or more of the label is derived from the incubation medium ($\frac{.0188}{.0232} \times 100 = 81\%$).

TABLE I

Density Difference of λ Phage Compared to

Unlabeled Light Marker Phage

Labeled frozen-thawed bacterial cells plus labeled incubation medium		Unlabeled frozen-thawed bacterial cells plus labeled incubation medium	
λ Temperate			
Experiment number	$\Delta\rho$ g/cc^3	Experiment number	$\Delta\rho$ g/cc^3
I	.018	V	.018
II	.026	VI	.025
III	.023	VII	.016
IV	.026	VIII	.016
Average	.0232 $\pm$ .003	Average	.0188 $\pm$ .004
λ Virulent			
Experiment number	$\Delta\rho$ g/cc^3	Experiment number	$\Delta\rho$ g/cc^3
IX	.017	XIV	.014
X	.027	XV	.027
Average	.022 $\pm$ .005	Average	.0205 $\pm$ .007

Differences in buoyant densities were estimated by plotting density as determined by density bead positions versus position of maximum phage concentrations.

A similar result is found in the case of λ virulent (Table I). If the incubation medium contains no isotope, a relatively light phage progeny results as indicated by the single peak when mixed with light marker phage (Fig. 1C).

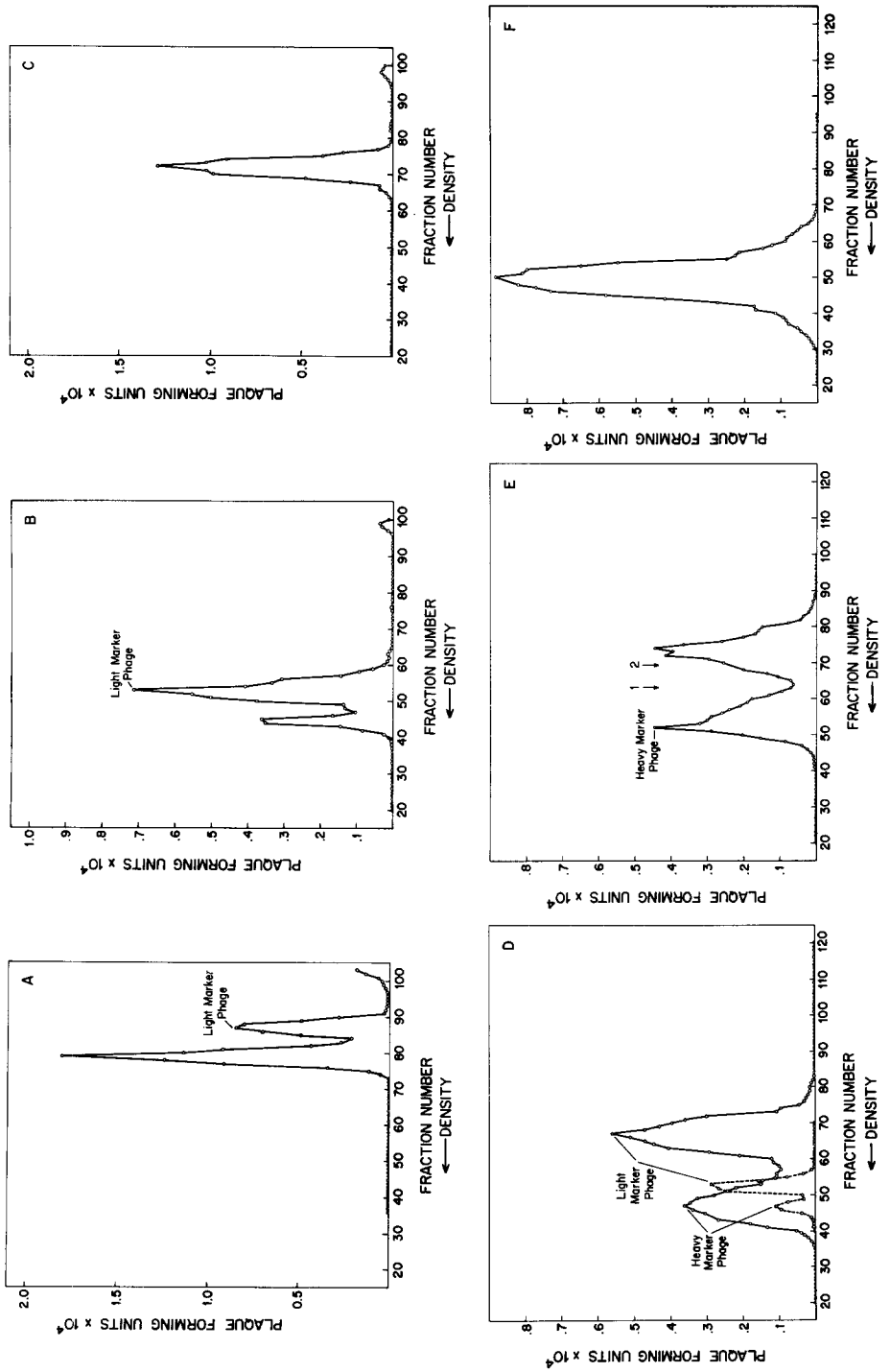
A
B
C
D
E
F
Light Marker Phage
Heavy Marker Phage
1
2
PLAQUE FORMING UNITS x 10⁴
FRACTION NUMBER
DENSITY

Fig. 1 Relative buoyant densities of phage in CsCl gradients.

A. Phage produced by infection of labeled frozen-thawed cells and labeled incubation medium with unlabeled DNA; 5 drop fractions collected.

B. Phage produced by infection of unlabeled frozen-thawed cells and labeled incubation medium with unlabeled DNA; 5 drop fractions collected.

C. Phage produced by infection of labeled frozen-thawed cells and unlabeled incubation medium with unlabeled DNA. Light marker phage in approximately equal amount was also added; 5 drop fractions collected.

D. Comparison of heavy and light marker phage. ——, 2 drop fractions collected; ----, 5 drop fractions collected.

E. Phage produced by infection of unlabeled frozen-thawed cells and unlabeled incubation medium with labeled DNA; 2 drop fractions collected. Arrow No. 1 indicates the approximate position where phage synthesized exclusively from density labeled infectious DNA would appear. Arrow No. 2 indicates the approximate position where phage containing 50% density labeled infectious DNA would appear.

F. Phage produced by infection of unlabeled frozen-thawed cells and unlabeled incubation medium with labeled DNA. Light marker phage in approximately equal amount was also added; 2 drop fractions collected.

These results indicate that a large proportion of the virus components must be synthesized de novo. The density increase in these experiments is comparable to the density increase when heavy marker λ virulent is prepared by growth in D_2O medium (ρ ranging from .0135 to .015 g/cc^3) (Fig. 1D). We can conclude therefore that in addition to phage protein a large fraction of the virus DNA must be newly synthesized. This comparison can be made since the additional ^{15}N label in the labeled frozen-thawed cells and incubation medium contributes only slightly to the total increased density.

These results are confirmed by the experiments in which density labeled DNA (isolated from heavy D-labeled marker phages) was used to infect unlabeled frozen-thawed cells and incubated in medium containing no isotope. Fig. 1E shows that under these conditions the newly synthesized virus is much less dense than the original heavy phage and the density difference is comparable to the difference between heavy and light marker phage (Fig. 1D). Since fractions of 2 drops were collected in these experiments, sensitivity of the method for detecting phage of intermediate densities was high. If the phage progeny had derived all of its DNA from the heavy infecting DNA, the resulting phage peak would have appeared approximately at the point marked by arrow No. 1 in Fig. 1E. Conservation of 50% of the DNA would have placed the progeny phage approximately at the point indicated by

arrow No. 2. Again, if progeny phage is mixed with light marker phage, a single peak results (Fig. 1F). The lower limit of detection of parental to progeny transfer in these experiments is of the order of 15%. The relative densities of the isolated density labeled DNA are shown in Fig. 2, indicating that heavy labeled infectious DNA was indeed adequately labeled. These experiments again demonstrate that in the infectious DNA frozen-thawed cell assay system completely new virus particles including DNA are synthesized.

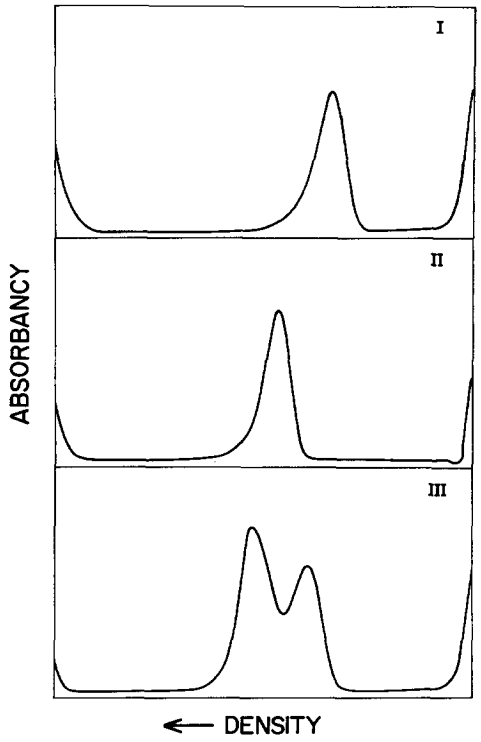

Fig. 2 Relative buoyant densities of infectious DNA.

I, unlabeled DNA; II, D-labeled DNA; III, equal mixture of D-labeled and unlabeled DNA.

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